Germination Capacity in Culture Medium of *Prosopis Laevigata* Seeds in the Presence of Copper Sulphate

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Abstract**—***Copper is a heavy metal that has been used as an anti-fungal agent in various crops, this is why it accumulates in certain agricultural lands at levels that become toxic to plants, as well as to microflora. Cooper, although essential to plants, is toxic when found in high concentrations. The objective of this study was to determine if this element is capable of stimulating and at the same time inhibiting germination of seeds of Prosopislaevigata (mesquite) depending on concentration of $CuS0_4.5H_20$ solutions. A completely randomized experimental design with seven treatments and three repetitions was used to determine tolerance of mesquite seeds to copper. The variables evaluated were percentage of daily germination (PDG-A, PDG-B, and PDG-C), accumulative germination (AG-A, AG-B, and AG-C), average germination time (AGT), germination rate (GR) and anhydrous weight (AW) of mesquite seeds. The culture media supplied with concentration of 10^{-4} M of copper sulfate ($CuS0_4.5H_20$), corresponding to treatment four (T_4) showed significant differences ($p\leq0.05$) in variable percentage of daily germination at 48 hours (PDG-B), which presented a germination of 66.7% in relation to treatment two (T_2) with a concentration of 10^{-2} M of $CuS0_4.5H_20$ and germination of 22.2%. Results obtained after 72 hours for percentage daily germination variable (PDG-C) with $^{>}p^{<0.1}$ showed that mesquite is a species that can tolerate and adapt in germination stage for culture medium with concentrations from 10^{-2} to 10^{-7}M of $CuS0_4.5H_20$, and consequently use of seedlings for phytoremediation of sites contaminated with copper.*

Keywords**— heavy metal, pesticide, germination percentage, average germination time, and culture medium.**

I. INTRODUCTION

Copper is a nutrient and traceable metal of prokaryotes and eukaryotes, since it is required in certain concentrations for metabolic functions (Navarrete *et al*, 2011), specifically, it acts as a cofactor in several enzymes and is required in several physiological processes (Auld, 2001;Cuypers*et al*., 2002; Nanda and Agrawal, 2016), and activates enzymes such as catalase, hydrogenase and cytochrome oxidase, also stimulates chlorophyll formation, intervenes in carbohydrate metabolism and oxidative processes, stimulates fixation of nitrogen and seed germination,(Yruela, 2005, 2009; Jelea*et al*., 2016). In addition, this element can influence each stage of plants cycle of life and their sensitivity to metals (Liu *et al*., 2005; Muccifona and Bellani, 2013).

Copper despite being an essential nutrient for plants, in high dose concentrations of 200 mg kg^{-1} is toxic for their development, *Leucaenaleucocephala*, it specially affects corn production. Which is one of the most important crops in Mexico. Chromium also, in high doses of 200 mg kg^{-1}affects the health of plants, although it was phytoextracted in a lower proportion than copper (Gardezi, 2007). However, copper is a pollutant among heavy metals in the environment (Zappala *et al.*, 2013), essential for plants in low concentrations, but toxic when these are high (Hattab*et al*., 2009; Hattab *et al*., 2010). Increase use of fungicides and pesticides allows accumulation of copper in soil (Yruela, 2005; Muccifona and Bellani, 2013; Jelea *et al*., 2016) triggered by various anthropogenic activities that include industry, agriculture, mining, transportation, urbanization, among others (Haque *et al*., 2009).

Copper stress can inhibit seed germination and subsequently plant growth (Nanda and Agrawal, 2016). Complete germination is a critical step since it requires activation of a complex regulatory system, which is controlled by intrinsic and extrinsic factors (Belwall *et al*., 2015). Reinoso *et al*., (2000), cited by Rios-Gomez *et al.*, (2010), found that germination is the most sensitive phase in *P. farcta, P. strombulifera and P. flexulosa*, since germination process begins with rapid water absorption (phase I), followed by an embryo expansion (phase II) and radicle germination(phase III). Germination and dormancy are influenced by environmental generic Jfactors to maximize long-term survival of seeds in many species (Koornneef *et al.*,2002; Belwall *et al*., 2015).

Therefore, the objective of this study was to examine the role of copper as a germination stimulator as well as its inhibitory effects of germination of mesquite (*Prosopislaevigata*) seeds, depending on concentration of $CuS0_4.5H_20$solutionsused. The variables studied were daily and accumulative germination, average germination time, germination rate and anhydrous weight of mesquite seeds. Mesquite has a variety of uses such as energy source, from firewood and coal; other important uses are elaboration of posts for fences, parquet, handicrafts, boards and planks, food for cattle, flour for human consumption, production of rubber and medicine (Solis, 1997); Prieto-Ruiz *et al.,* 2013). In addition, its ecological importance lies in its ability to fix atmospheric nitrogen, which enriches soil around it and promotes the contribution of nutrients (Quiñonez-Gutiérrez *et al.,* 2013). Finally, according to what was established by Clemens *et al*., (2002), these trees are ideal in phytoremediation projects since they have multiple uses and can adapt to particular environmental conditions in their habitat. An example is their conditioning to sites with high concentrations of heavy metals and contribution as a defense against herbivores and pathogens (Boyd and Martens, 1998; Martens and Boyd, 2002; Tolra *et al*., 2001; Barceló and Poschenrleder, 2003).

II. MATERIALS AND METHODS

Seeds, were collected directly from mesquite trees in September 2016, from the lower watershed of Tulancingo River, municipality of Acatlán, Hidalgo, Mexico. These were mechanically extracted from mesquite pods by using conventional tweezers. Seeds not damaged by insects were selected. In addition, a preliminary germination test with n = 30 seeds indicated that it averaged 30% (Table 1).

In the laboratory, 315 homogeneous seeds in dimensions and weight were selected (Table l). These were immersed in 45 ml of sulfuric acid for 3 minutes as suggested by D'Aubeterre *et al*., (2002) and Madueño-Molina *et al*., (2006). They were subsequently washed with distilled water for a period of 5 minutes.

In twenty-one Petri dishes fifteen seeds and 4 ml of sulphate dilution of $CuS0_4.5H_20$ and control treatment were added per box. These were prepared by adding 13.5 ml of distilled water and 1.5 ml of $CuS0_4.5H_20$ in flasks with a capacity of 20 ml, which were supplied in the following concentrations: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M. The control treatment consisted in adding 4 ml of distilled water. Subsequently, the Petri dishes were placed in a germination incubator at a constant temperature of 28-30^{0}C for 72 hours.

TABLE 1
MORPHOLOGICAL CHARACTERISTICS OF SEEDS OF *P. laevigata*.

Feature	N=315
Shape	Rhomboid-flattened
Color	Brown-yellow
Length (mm)	6.52±0.1 1
Width (mm)	4.53±0.08
Weight of 100 seeds (g)	4.8
Preliminary germination (%)	30±16

Table 1. Morphological characteristics of *Prosopis laevigata* seeds, Feature N = 315, Shape =Rhomboid-flattened, Color = Brown-yellow, Length (mm) = 6.52±0.1 1, Width (mm) = 4.53±0.08, Weight of 100 seeds (g) = 4.8, Preliminary germination (%) = 30±16.

Once germination process was completed, determination of anhydrous weight of germinated mesquite seeds was carried out in the Animal Nutrition Laboratory of the Postgraduate College, by introducing 21 experimental units in a drying oven for 72 hours until constant weight acquisition and subsequent introduction in desiccators with active silica for 15 minutes, followed by a subsequent weighing in an analytical balance.

Seeds were considered germinated when germination of radicle was observed (Jamal *et al*., 2006). The variables studied were percentage of daily germination (PDG) every 24 hours and accumulative germination (AG), germinationrate (GR), and average germination time (AGT) which were determined according to the methodology of Muccifona and Bellani, (2013); Belwall *et al*., (2015).

$$PDG = \frac{N}{TNS} * 100$$

$$GR = \sum \frac{NSG}{d}$$

$$AGT = \left| \sum (n \times h)/N \right|$$

Where **TNS** is total number of seeds used, **NSG** is number of seeds germinate deach day, **d**are the days for germination, **n** is the number of seeds germinated at a given time, **h** time after inhibition in hours in which germination occurred and **N** total number of seeds germinated in incubation period. In addition, an hydrous weight of seeds was determined.

A complete randomized experimental design with seven treatments and three repetitions was used to determine tolerance of mesquite seeds at different copper concentrations. Data was subjected to tests of homogeneity variances, Bartlett and distribution normality, Shapiro-Wilk. When data showed a normal distribution, Analysis of Variance (**ANOVA**) and comparison of Tukey means ($p \leq 0.05$) were used to compare treatment means. Non-normal data was submitted to non-parametric test Kruscal-Wallis and comparison means of Mann-Witney (da Trindade-Lessa *et al*., 2015).

III. RESULTS AND DISCUSSION

Brooks *et al*., (1998) and Khan *et al*., (2000), cited by Zappalà *et al*., (2013), and mentioned that there are more than 450 species considered as hyper accumulators of various metals. By definition, they are herbaceous or woody plants that accumulate and tolerate high concentration of metals in their stems without visible symptoms, compared to those found in non-accumulative plants (Barceló and Poschenrleder, 2003). Unlike herbaceous plants, mesquite trees are woody plants that prove to be ideal in remediation of heavy metals since they can tolerate high concentrations of pollutants due to their greater biomass, deep roots, and ease of harvest. In addition, they tolerate and accumulate a wide range of heavy metals in the aerial and usable sections. In addition, trees have other advantages such as soil stabilization, erosion prevention and minimization of pollutant expansion due to their perennial presence (Clemens *et al*., 2002; Paz-Alberto and Sigua, 2013). Rios-Gomez *et al*., (2010) mentioned that (*P. laevigata*) can be classified as a candidate species to be used for soil rehabilitation purposes.

TABLE 2
PERCENTAGES OF DAILY GERMINATION OF (*P. laevigata*) SEEDS AT CONCENTRATIONS OF $CuS0_4.5H_20$.

Treatment Conditions		PDG-A (%) *	PDG-A (%) **	PDG-B (%) *	PDG-B (%)	PDG-C
(Control)	Distilled water					
T_1		51.1 a	ab	40.0 ab	abc	4.4 a
T_2	10^{-3} M	71.1 a	ab	22.2 b	c	4.5 a
T_3		35.6 a	ab	62.2 ab	ab	2.2 a
T_4		26.7 a	b	66.7 a		
T_5		60.0 a		40.0 ab	abc	0.0 a
T_6	10^{-7} M	75.6 a	ab		bc	0.0 a
T_7		57.8 a	ab	42.2 ab	abc	0.0 a

PDG-A, PDG-B, and PDG-C = percentage daily germination at 24, 48 and 72 hours. PDG-C: means with different letters in same column are statistically different (Mann-Whitney) test. Means with same letter in same column are statistically the same (Tukey, $p \leq 0.05$ and/or 0.1).* $p \leq 0.05$, ** $p \leq 0.1$

It is essential for phytore mediation purposes to select the right seeds for planting production, since these can follow genetic mechanisms of tolerance to heavy metals according to their origin (Haque *et al*., 2009). A decrease in germination capacity is related to increase in concentration of metals such as copper, which reduces root growth and consequently aerial growth of plants (Jelea *et al*., 2016). In this sense, it is well documented that seeds germination process is highly affected by heavy metals stress (Ahsan *et al*., 2007). For example, Carrillo-Castañeda *et al*., (2002) pointed out that toxic effect of pollutants on seeds germination is a good indicator of a possible tolerance of plants against these pollutants.

During germination, imbibitions of seeds are very important, since metabolism is quickly reactivated with respiration, as well as enzymatic activity and organelles. In addition, ribonucleic acid (RNA) and protein synthesis are fundamental cellular activities involved in germination and preparation for consequent growth (Bewley, 1997; Gallão *et al*., 2007). In this study, preliminary seed test indicated presence of dormancy. However, use of H_2S0_4 allowed hydrophobic layer to wear, and therefore imbibitions of water, which favored completion of dormancy followed by subsequent germination (Gallão *et al*., 2007) in media with different concentrations of $CuS0_4.5H_20$. In fact, effect of sulfuric acid (H_2S0_4) concentrated in the genus *Prosopis* at germination has been reported by D'Aubeterre *et al*., (2002), quoted by Madueño-Molina *et al*., (2006) who found that their use improved germination of *P.laevigata* and *P. glandulosa* seeds (Villarreal-Garza *et al*., 2012).

The effects of different dilutions of copper sulphate ($CuS0_4.5H_20$) on germination of mesquite seeds (*P. laevigata*) in this study only showed significant differences ($p \leq 0.05$) in variable percentage of daily germination at 48 hours (PDG-B) treatment four (T_4) with a germination of 66.7% (10^{-4} M) in relation to 22.2% corresponding to treatment two (T_2) with concentration of 10^{-2} M of $CuS0_4.5H_20$ (Table 2 and Figure 1), followed by 100% germination (PDG-C) for all treatments at 72 hours. This indicated a temporary inhibitor effect of copper on the germination of mesquite seeds. Similar results in germination process were observed by Muccifona and Bellani, (2013), who observed that control treatment and $CuBr_2$ concentration of 10^{-3} M in *Vicia Sativa* reached 100% germination in 48 hours of imbibitions, while 5 x 10^{-3} M treatment with $CuBr_2$ showed an inhibitory effect 30 hours after imbibitions, with a subsequent germination of 100% at 72 hours.

TABLE 3
CUMULATIVE GERMINATION OF *Prosopieslaevigata* SEEDS IN SOLUTION OF $CuSO_4.5H_2O$

Treatment	Conditions	GA-A (%) *	GA-A(%) **	GA-B (%) ***	GA-C (%) ***
T_1 (control)	Distilled water	51.1a	ab	91.1a	95.5a
T_2	10^{-2} M	71.1a	ab	93.3a	97.8a
T_3	10^{-3}M	35.6a	ab	97.8a	100.0a
T_4	10^{-4}M	26.7a	b	93.3a	93.3a
T_5	10^{-5}M	60.0a	ab	100.0a	100.0a
T_6	10^{-6}M	75.6a	a	100.0a	100.0a
T_7	10^{-7}M	57.8a	ab	100.0a	100.0a

GA-A, GA-B, GA-C = germination accumulated at 24, 48 and 72 hours. GA-C: means with different letters the same column is statistically different (Mann-Whitney) test. Means with same letter and column are statistically the same (Tukey, p ≤.05 $^{and}/_{or}$ 0.1). $^*p \leq 0.05$, $^{**}p \leq 0.1$.

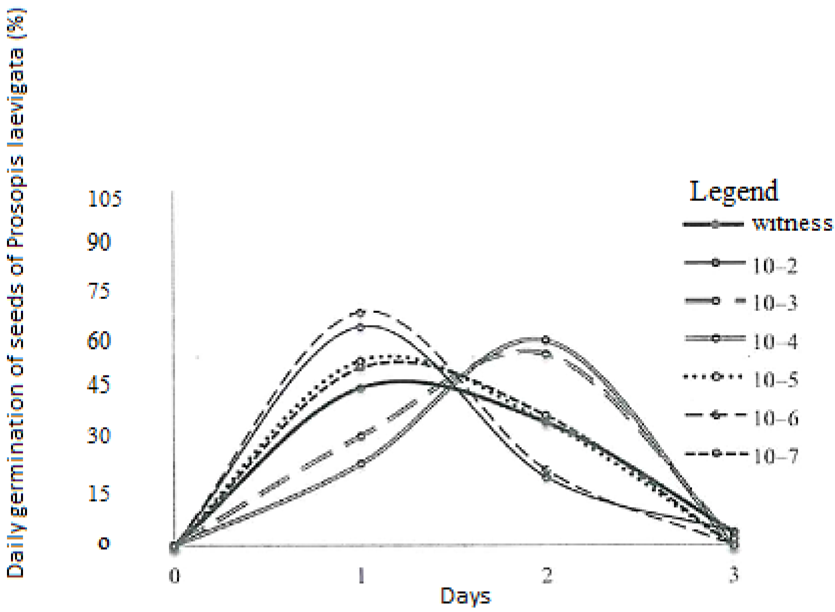

FIGURE 1. Percentage daily germination of *P. laevigata* in solution of molar concentration of sulphate $CuS0_4$. $5H_2$

In relation to eight remaining variables that showed no significant differences for $p^{>0.05}$ in solutions of 10^{-2} M to 10^{-7} M (T_2-T_7)of $CuS0_4.5H_20$ and control treatment (T_1), percentage daily germination at 24 and 72 hours (PDG-A and PDG-B); accumulative germination at 24, 48 and 72 hours (AG-A, AG-B, and AG-C) (Table 2-3, and Figure 1-2); average germination time (AGT); germination rate (GR); and anhydrous weight (AW) of seedlings (Table 4) 4 days for mesquite, results coincided with what was established by Chaignon and Hinsinger (2003), who mention that germination is relatively insensitive to several toxic substances, because at this stage seedlings use their reserves (Zappalà *et al*., 2013) and, therefore, there is lower probability that metal ions interfere until germination process is completed (Stefani *et al*., 1991; Street *et al*., 2007). Likewise, results in this study are consistent with those found by Zappalà *et al*., (2013), who used copper concentrations of 0, 50, 100, 200, 300, 400, 500 and 600 ppm in their experimentation, which did not affected seeds germination of *P. pubescens*.

However, significant differences were observed with $p^{<0.1}$ for percentage daily germination variable at 24 hours (PDG-A) and 48 hours (PDG-B), accumulated germination at24 hours (AG-A) and average germination time (AGT) (Tables 2-4 and Figures 1-2). This indicated that at 24 hours, both percentage daily germination (PDG) and accumulated germination (AG-A), significant differences were observed in germination rate for treatmentsix (T_6) in relation to treatment four (T_4). In addition, percentage daily germination (PDG-B) at 48 hours showed significant differences for treatment four (T_4) in relation to treatment two (T_2). This temporary inhibitor effect is consistent with that observed in saline media by Rios-Gomez *et al*., (2010), who reported that germination of *Prosopis spp*. is more sensitive to sulfate ions such as Na_2S0_4 than to NaCl. Finally, in variable average germination time (AGT) a significant increase was observed p<0.l of speed of this physiological process for mesquite seeds included in treatment six (T_6) in relation to treatment four (T_4) (Table 2-4 and Figure 1-2), which suggests that plants can adopt various strategies in presence of metals in their environment (Baker 1981; Barceló, J. and Poschenrieder, C. 2003; Ruiz- Huerta and Armienta-Hernandez, 2012).

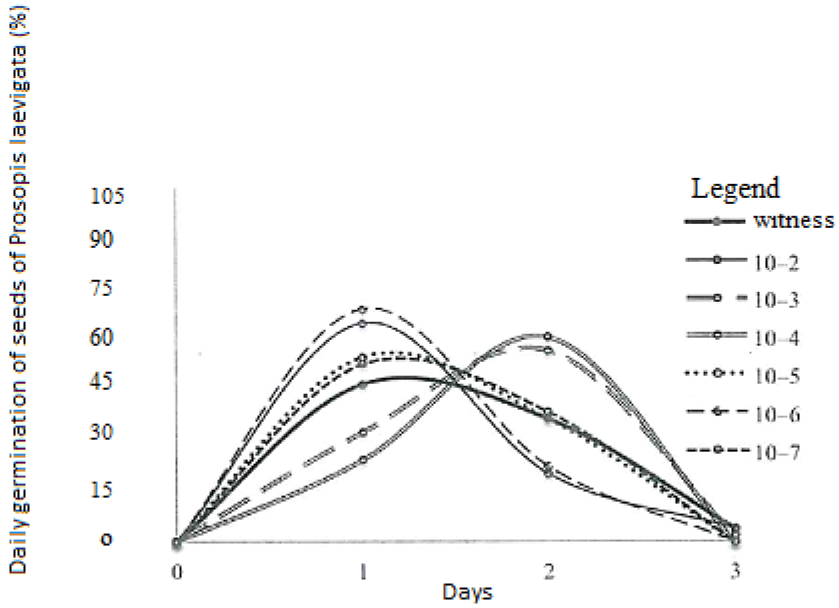

FIGURE 2. Cumulative seed germination data (%) *P. laevigata* in presence of molar concentrations of $CuS0_4.5H_20$ solutions.

The results obtained after 72 hours for 0.059 >0.1 (Table 2-4) contrast with the fact that copper strongly affects germination percentage of *Fabaceae* such as *P. pubescens* (Navarrete *et al*., 2011), *Medicago sativa* (Peralta *et al*., 2001), *Pisumsativum* (Mihoub *et al*., 2005) and *V. faba* (Olivares *et al*., 2015). Likewise, they also differ with those obtained by Nanda and Agrawal (2016) in an experiment with copper concentration of 0, l, 10, 50, 100, and 200 mgL^{-1}, who observed that *Cassia angustifolia* seeds showed a decline in germination initiated with 1 mgL^{-1} and a maximization of 200 mgL^{-1}. They concluded that germination was sensitive to external environment, which is regulated by changes in state of cellular oxidation-reduction; therefore, addition of metals aggravates micro environment and causes damage to proteins that leads to a reduction in germination (El-Maarouf-Bouteau and Bailly, 2008). In this sense, Street *et al*., (2007) mentioned that although species belong to the same family, germination response in presence of copper mark differently.

Finally, results obtained in this study are subject to compliance with the assumptions of complete randomized experimental design (Gutiérrez-Pulido and de laVara Salazar, 2008): 1) errors are independent, 2) errors are normally distributed with zero means and constant variance, 3) existence of homogeneity of variances between treatments, and 4) model has linear and additive effects (Lopez-Bautista and Gonzalez-Ramirez, 2014). By default, non-normal data subordinated to non-parametric test Kruscal-Wallis and comparison means of Mann-Witney (da Trindade-Lessa *et al.,* 2015). However, Mandeville (2012) mentions that unknown effects on variable response are called experimental errors (George *et al*., 2005; Gill, 1978), and some methods to reduce them are, for example, homogeneous and random selection of experimental units, increase number of repetitions, among others (Gill, 1978). This allows possible environmental and temporary effects to be distributed equally among treatments (Gutiérrez-Pulido and de laVara-Salazar, 2008). In addition, among the disadvantages of this experimental design based on principles of repetition and randomization, Lopez-Bautista and Gonzalez-Ramirez (2014) point out that because sources of variation are not associated with treatments included as a random variation residue, good analysis and accuracy can be compromised.

TABLE 4
AVERAGE GERMINATION TIME, GERMINATION RATE AND ANHYDROUS WEIGHT OF *P. laevigata* SEEDS IN $CuS0_4.5H_20$ SOLUTIONS.

Treatment	Conditions	AGT (h) *	AGT (h) **	GR (seeds/day) * **	AW (Mg) * **
(Control)	Distilled water				
T_1	10^{-1}M		ab	6.4 a	449.2 a
T_2	10^{-2}M	31.7 a	ab	5.7 a	467.4 a
T_3	10^{-3}M	40.0 a	ab	6.7 a	474.0 a
T_4	10^{-4}M	41.3 a	a	7.0 a	409.4 a
T_5	10^{-5} M	33.6 a	ab	7.5 a	472.9 a
T_6	10^{-6} M	29.9 a	b	7.5 a	485.7 a
T_7	10^{-7} M	34.0 a	ab	7.5 a	470.0 a

Control-treatment with distilled water, and 10---10—dilutions of CuS04.5H20. Means with same letter in same column are statistically the same (Tukey, p 0.05). AGT = average germination time, GR = germination rate, and AW = anhydrous weight. Means with same letter in same column are statistically the same (Tukey, p 9.05 and/or 0.1) $*p\leq0.05$, $^{**}p\leq0.1$.

IV. CONCLUSION

(*P. laevigata*) is a species that can tolerate and adapt in germination stages to culture media with solutions of $CuS0_4.5H_20$ from 10^{-2} M to 10^{-7} M, according to results obtained using an experimental design and assumed assumptions of them. In addition, use of $CuS0_4.5H_20$ showed temporary effects during germination process, without showing significant effects (0. 05≤p≥0.l) on percentage daily germination (PDG-C) at 72 hours. Therefore, mesquite seeds are recommended for seedling production and for phytore mediation of sites contaminated with copper. Although, it is suggested that future greenhouse and field experiments be done in order to investigate different adaptation strategies of this species against the presence of metals in its surroundings.

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